0022-202X/81/7705-0385$02.00/0
THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 77:385–388, 1981

Vol. 77, No. 5
Printed in U.S.A.

Cyclic Nucleotide Content of Passaged Keratinocytes in Culture During Various Growth Stages

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Human newborn foreskin keratinocytes were maintained in culture in first passage for up to 60 days. An initial lag phase was followed by a growth and a final plateau phase. During this time, cell numbers, the morphologic apperance of both attached and detached cells, DNA, and cyclic nucleotide content were evaluated. Cell cycle analysis during growth phase was performed by flow cytofluorometry.

High levels of cAMP and cGMP coincided with the early growth phase, when up to 70% of cells were in S, G_2, or M compartments of the cell cycle. Halfway through growth phase, a fall in cyclic nucleotide levels coincided with a drop in mitotic index and increase in G_1 cells. Plateau phase was characterized by increased desquamation, a gradual elevation of intracellular cAMP, cGMP, and DNA, and in later stages, an increased proportion of squamous cells in the attached layers. The oldest cultures were mostly squamous with low cyclic nucleotide levels.

Results suggest that high cyclic nucleotide content is a feature of cycling, possibly S phase keratinocytes. Onset of differentiation coincides with a small but definite increase in both cAMP and cGMP, which in general vary in the same direction.

Recently, the serial culture of human keratinocytes obtained from adult (mastectomy, autopsy) skin or newborn human foreskin has been described [1,2]. In their first passage, and after a period of several days, foreskin keratinocytes pass through a growth phase until a confluent plateau phase is achieved when keratinization markers become evident [2]. The exponential increase in cell number, and apparent homogeneity under light microscopic examination, support the idea that a high proportion of cycling cells exists during the first half of the growth phase. In contrast, primary cultures are characterized by focal points of keratinization with peripheral proliferation. If primary cultures are used in experiments, this heterogeneity precludes satisfactory interpretation of biochemical data.

For some time, the significance of the cyclic nucleotides (CNs) adenosine-3′,5′-monophosphate (cAMP) and guanosine-3′,5′-monophosphate (cGMP) for the proliferation and maturation of keratinocytes has been the focus of much investigation. Data obtained mainly from studies of psoriasis indicate possible changes in cAMP and cGMP in a mitotic epidermis, but since epidermal samples contain keratinocytes in all stages of differentiation, it is difficult to relate observed changes in CNs to growth or maturation.

Manuscript received February 22, 1980; accepted for publication April 5, 1981.

This work was initially reported at the Fortieth Annual Meeting of the Society for Investigative Dermatology, Washington D.C., May, 1979.

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Abbreviations:
cAMP: adenosine-3′, 5′-monophosphate
cGMP: guanosine-3′, 5′-monophosphate
CNs: cyclic nucleotides

This is a study of the CN content of first passage human foreskin keratinocytes in culture, when growth and maturation occurred during different stages.

MATERIALS AND METHODS

Cell Culture

First passage keratinocytes were prepared from primary cultures of human newborn foreskin cells as described [2]. These were maintained at 37°C in 5% CO_2 humidified conditions, using 3.5 cm dishes with thin-gel collagen [1], and Dulbecco MEM with 20% fetal calf serum, gentamicin (50 μg/ml) and mycostatin (10 U/ml). One series of dishes received medium containing hydrocortisone (HC;0.4 μg/ml) to which was added mouse epidermal growth factor (mEGF:10 ng/ml) on day 5 onwards; a second series used medium without either agent. Media were changed every 3 days.

Cell Harvest

On selected days, just prior to a medium change, medium plus detached cells (which had accumulated since the last change) were removed from duplicate dishes. The cells were suspended by pipetting and counted in a hemocytometer. The attached cells were rinsed with PBS and removed using trypsin (0.3%) and EDTA (1.0%) in PBS (Ca^{++}, Mg^{++}-free; 1.0 ml) for 10 min at 37°C. Dishes were rinsed with an additional volume of PBS (1.0–2.5 ml) and the trypsin suspensions plus washings gently pipetted to disperse the cells. A small aliquot was used for enumeration, and an equal volume of TCA (10%) added to the remainder. The precipitate was collected by centrifugation and DNA determined fluorometrically [3].

Cyclic Nucleotide Radioimmunoassay

Duplicate dishes were selected prior to a medium change, the medium aspirated and cold 5% TCA added. These were stored at −20°C until required, Cyclic AMP, cGMP and DNA were assayed as described [4].

Determination of Mitotic Index

Duplicate dishes were selected and the medium replaced with fresh medium (2 ml) containing vinblastine (0.05 ug/ml) for 4 hr at 37°C. At this time, the medium was removed, the cell layer rinsed with PBS, fixed in acetic acid/alcohol (1:3 v/v) for 10 min, rinsed thoroughly with water, air dried and hematoxylin (2 ml) added. After 10 min the layer was rinsed with water, air dried and covered with a film of Hydramount. The number of cells in prophase in 50 randomly selected microscope fields was noted (mean = $\bar{x}$). The mitotic index per 10^3 cells was expressed as $1000\ \bar{x}\ R/t$, where R is the ratio of the area of a 3.5 cm dish to the area of the microscope field, and t is the total number of cells per dish (from parallel cultures-see under "Cell Harvest"). In early stages of experiments when cells had partially covered the dish surface, the index was calculated using $1000\ \bar{x}/t'$ where t' is the mean number of cells in a field.

In one experient, first passage cells were grown on thick (2 mm) collagen gels which have been described [1]. After exposure to vinblastine and rinsing, selected dishes were treated with Zenkers solution to harden the gels, and then 6-mm biopsies punched through the cell layers and gel at selected areas of the cultures. After alcohol dehydration, the biopsies were infiltrated with paraffin, sectioned and stained (H and E) and examined by light microscopy.

Flow Cytofluorometry

In a further experiment, at various times during growth phase, keratinocytes were removed from duplicate dishes by trypsin, fixed with ethanol, treated with ribonuclease, and stained with propidium iodide as described by Hawkes and Bartholomew [18], for flow micro-

fluorometric analysis. The processed signals from at least 10^6 cells were accumulated and displayed as frequency distribution histograms, and the data analyzed by computer.

Materials

Tissue culture supplies were obtained from Grand Island Biological and Microbiological Associates. Serum was obtained from Flow Laboratories. Biochemicals were from Sigma, and trypsin from Nutritional Biochemicals. EGF and RIA supplies were purchased from Collaborative Research. For vinblastine, we used Velban (Lilly). Hydramount was obtained from Biomedical Specialities, Santa Monica, CA 90406. Hematoxylin solution was according to the Harris formula, prepared by Anderson Laboratories Inc., Fort Worth, Texas, 76112.

RESULTS

Growth Characteristics

Representative growth curves are shown in Fig 1 *A*, 2 *A*, and 3 *A*. Results from 6 experiments showed that passaged keratinocytes passed through an initial lag phase of 3–7 days, the length depending in part on the presence of HC-mEGF (see below). This was followed by a logarithmic growth stage (usually during days 5–15). Entry into the plateau phase was accompanied by a rapid rise in detachment of cells of squamous configuration into the medium (Fig. 1 *A*). During the plateau phase the numbers of attached cells declined (Fig 1 *A*, 2 *A*).

After the lag phase, attached cells, after trypsin release from the dishes, were predominantly round and translucent and formed 86–95% of the population, on microscopic evaluation. After about day 35 relative porportion of squamous cells increased, to about 80% at day 49 (Fig 2 *A*). Cells detaching into the medium were almost always squames, and exhibited a higher ratio of histidine-leucine incorporation into protein, as shown in an earlier report [2] and confirmed here (data not shown).

Mitotic Index

During early growth phase, an intense wave of mitotic activity was observed using vinblastine arrest. About 20% of attached cells were in prophase on day 5, and smaller number on day 8 (Fig 2 *A*) while maximum DNA occurred on day 7 (Fig 2 *B*). Flow cytofluorometry confirmed these data (Fig 2 *C*); about 30% of cells were in G_2+M on day 5 and 20% on day 8, while 60% were in S on day 7. Mitotic activity fell sharply about halfway through growth phase, and several minor periods of mitoses were observed later, at days 17–25 and 33–39 (Fig 2 *A*).

The very low mitotic indices observed using vinblastine after the initial wave were not due to concealment of mitoses by overlying cells. Sectioning of cultures older than 10 days showed the presence of 1 or 2 layers of flat, transparent cells which did not obscure basal mitoses. Cultures receiving HC-mEGF formed characteristic "whorls" with well-demarcated central foci of 8–10 layers whose basal strata were devoid of mitotic activity.

DNA Determinations

The DNA content per dish rose during growth phase and remained roughly constant during plateau phase (Fig 1 *A*). DNA content per cell showed a peak which coincided with S phase cells (Fig 2 *B*). After this, values per cell tended to fluctuate, but in general they showed an overall increase with time.

CN Assay Results

Routine validation of the RIA methods used for cAMP and cGMP have been discussed elsewhere [4]. Data from representative experiments are shown in Fig 1 B, C, and 3 B, C.Levels of both CNs were high during the lag phase but rose to their maximum values during the period of greatest mitotic activity, before falling sharply. Lowest levels of both CNs were obtained towards the end of the growth phase. During the plateau phase, cAMP decreased while cGMP levels remained fairly constant when DNA was used as data base (Fig 1 *B*). However, when expressed in terms of cell number, both CNs showed a slow increase starting at about day 22 (Fig 1 *C*). After about day 45, CN levels fell off regardless of the data base.

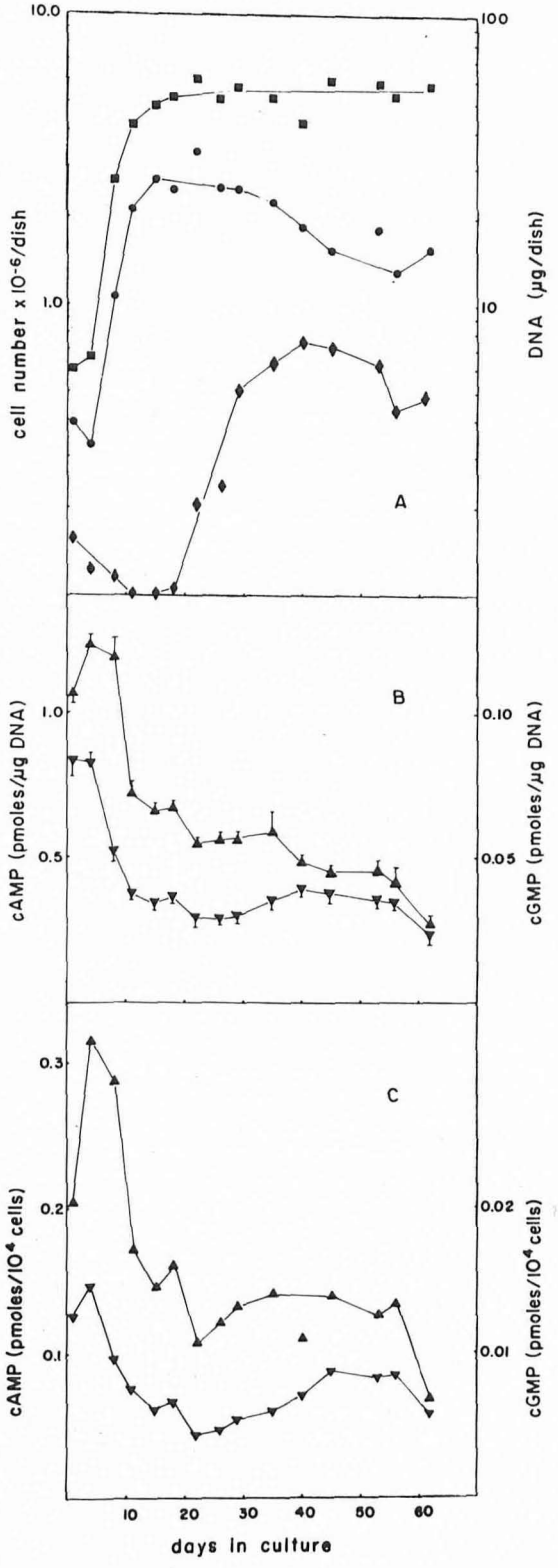

FIG 1. *A*, Variation with time in the number of attached cells (●), the number of detached cells accumulating in 3 days (◆), and DNA per dish (■). Values are means from 2 dishes. These patterns have been observed in many experiments. *B* and *C*, cAMP (▲) and cGMP (▼) content of attached cells in terms of DNA (*B*) and cell number (*C*). Values in *B* are means ± SD from 3 dishes. Cells were grown with HC-mEGF. These patterns have been observed in 4 experiments.

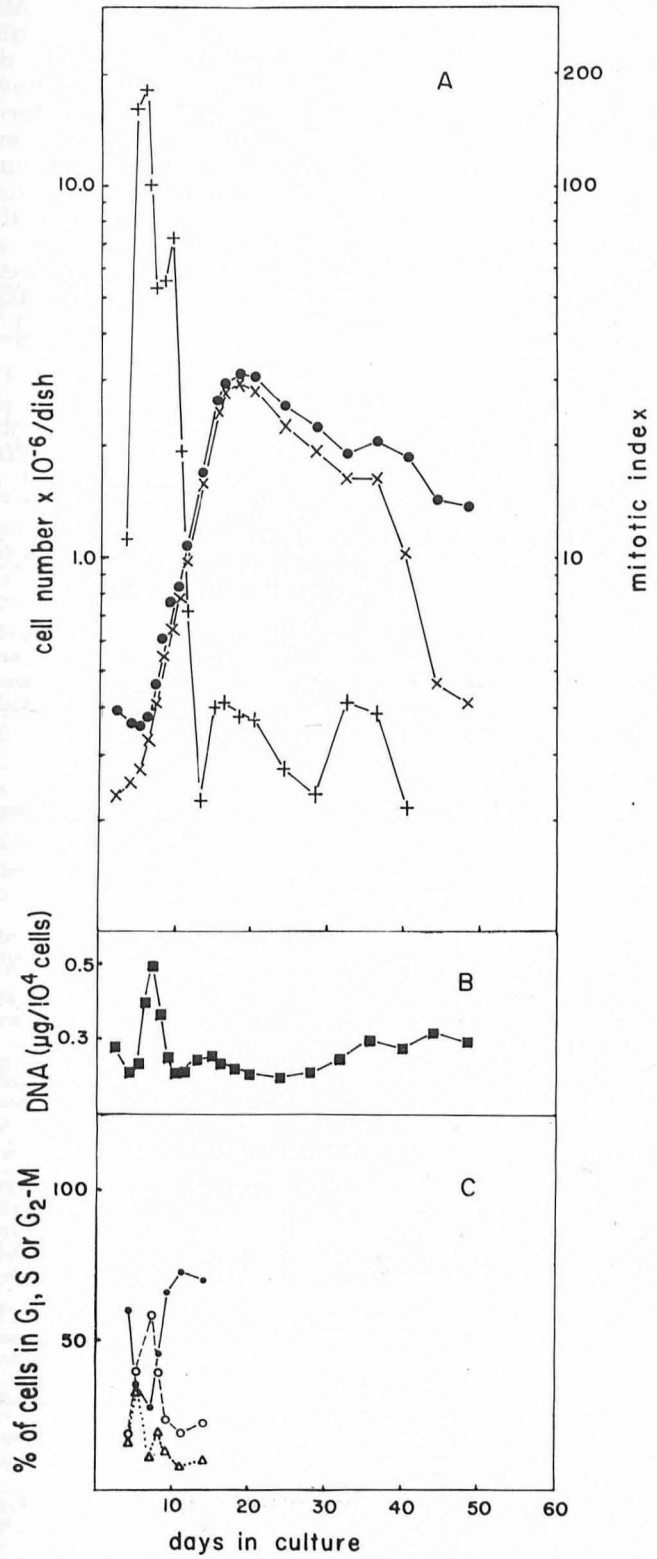

FIG 2. *A*, Representative data showing variation in number of attached cells (●), number of round translucent cells obtained by trypsinization at each time point (×), and mitotic index (+). *B*, DNA content per 10^4 attached cells. *C*, Distribution of cells in G_1 (●), S (○), and G_2 + M (△) at various times during the growth phase. In A, B, and *C* all values are means of duplicate dishes in one experiment. All cells were grown with HC-mEGF.

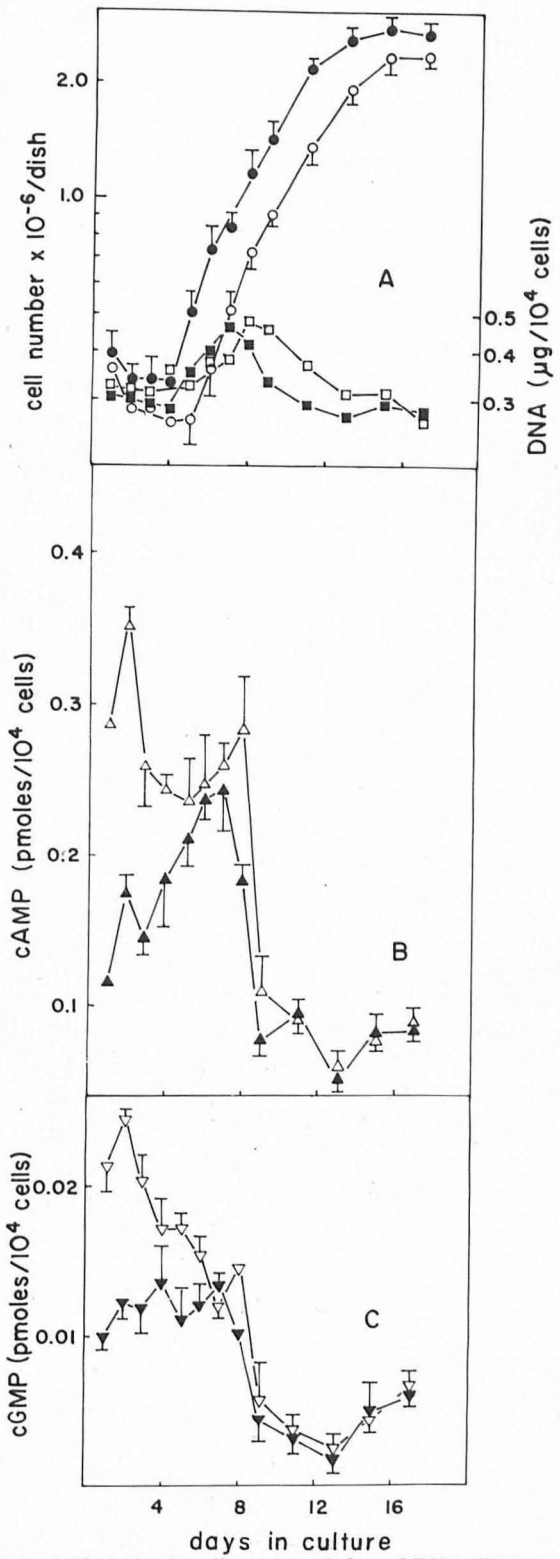

FIG 3. *A*, Variation in cell number (● ○) and DNA (■ □) during lag and growth phases. Values are means from 4 dishes (2 experiments). *Solid symbols* indicate cells grown with HC-mEGF; *open* symbols without. *B* and *C*, Effect of culture conditions on cAMP (*B*) and cGMP (*C*) content of attached cells. Solid symbols (▲▼) indicate dishes receiving HC-mEGF; open symbols (△▽) receive neither. Points are means ± SD from 4 determinations, from 2 experiments.

Effect of HC-mEGF on Growth and CN Levels

The data presented in Fig 1 and 2 were obtained with cells grown in the presence of HC-mEGF, and in Fig 3 with cells cultured with and without these reagents. The lag and growth phases were examined in detail using cells harvested daily (Fig 3). Cells plated out in the presence of HC showed greater plating efficiency. These showed lower CN levels during the lag phase than cells not receiving HC, and these levels tended to rise (Fig 3 *B*, *C*) to a peak at day 7 when DNA content was also

maximal (Fig 3 *A*). On day 5 and thereafter mEGF was added routinely to media containing HC. Addition of mEGF seemed to initiate growth phase, while the control cells (no HC-mEGF) entered growth phase about 2–3 days later, with CN and DNA peaks on day 8 (Fig 3 *A*, *B*, *C*). However, in both control and treated cells, early CN peaks on days 2 and 4 could not be correlated with the cell cycle (Fig 3 *B*, *C*).

Shortly after CN levels in both cell populations fell, and were identical afterwards, with similar growth rates.

DISCUSSION

These studies confirm and extend earlier observations on the growth patterns of passaged foreskin keratinocytes [2]. A growth phase was observed with an 8- to 10-fold increase in cell number. During the first half of this phase a high proportion of the cells appeared to be cycling, followed by a sharp reduction in mitotic index. Thereafter the cells reverted to specialization; mitotic index never exceeded 4.0 and desquamation sharply increased. It seems that detachment occurs when the keratinocyte reaches a certain point in its maturation process, although attached squamous cells become increasingly prominent in cultures older than 35 days.

Newly plated keratinocytes possess elevated cAMP levels probably as the result of their exposure to trypsin and mechanical trauma encountered during plating. These levels tended to subside after about 3 days, but were again raised to maximum amounts during early growth phase and coincided with the period of greatest DNA content. This suggests that a population of cycling keratinocytes has higher CN levels than in other stages of maturation. Lowest levels of CNs were observed during the latter part of the growth phase. In an earlier report these low levels were equated with growth [5], but this conclusion should be clarified: although cell number doubled during the second half of the growth phase, mitotic activity was lower than in the first half.

Studies of the fluctuations of cAMP and cGMP in synchronized cells have been carried out using various cell types [6,7]. In the present experiments, an obvious degree of synchronous growth existed during the first few days. The data suggested that S phase keratinocytes may have elevated CN levels, but this conclusion must remain tentative until demonstrated with a deliberately synchronized population maintained in culture long enough for the fluctuations in CNs observed after plating and during lag phase to subside. These fluctuations were particularly evident around days 1–3 in cells plated without HC, which seemed to suppress higher (lag phase) levels.

If the peaks in CNs observed on days 7 and 8 indeed represent S phase cells, one would expect that the psoriatic epidermis with many cycling keratinocytes would have a higher content of cAMP than healthy epidermis. This has indeed been reported although the evidence on this point is conflicting [8].

In plateau phase, amounts of CNs per cell increased, together with the relative numbers of attached squamous cells. In passing, it should be emphasized that CN levels per cell were calculated using the cell number in parallel dishes, as it is not feasible to obtain meaningful data on the CN content of cells treated with trypsin/EDTA for enumeration. However, several experiments gave consistent results. One might expect the CN content of detached cells to be high, but it is difficult to interpret this data for cells collected by centrifugation. It is possible that detached cells relinquish part of their CN content to the medium; this is suggested by the final decrease in CNs of attached cells after 55 days in culture, and by a report that the cAMP of isolated keratinocytes falls with differentiation [9]. Measurement of total CN levels in detached cells plus medium may provide more information on change in CNs during differentiation.

Increase in cAMP is usually associated with the induction and maintenance of differentiation [10]. Several lines of indirect evidence suggest that this is true for epidermis; treatment of keratinocytes with reagents which elevate cAMP levels evoke the appearance of markers of keratinization [11]. In contrast, there is hardly any evidence for the involvement of cGMP. Moryama has suggested a negative role for cGMP in the differentiation of myoblast cultures, when cGMP necessarily decreases while cAMP increases [12]. Recently, Zwiller has shown that both dibutyryl cAMP and the corresponding cGMP derivative can induce morphologic differentiation of neuroblastoma cells in culture, but the most striking evidence (neurite formation) resulted when both agents were supplied simultaneously [13]. In the present study, cAMP and cGMP levels ran parallel, both increasing in aging cultures between days 21 and 42. Marcelo has found that 8-bromo cGMP enhanced the synthesis of specialized proteins and the stratification of cultured keratinocytes [14]. These data suggest that cGMP is involved in differentiation.

In terms of growth, the only apparent effect of mEGF was to shorten the lag phase. Addition of mEGF to HC-treated cells on day 5 did not affect CN levels, but initiated the growth phase. mEGF was not added earlier due to toxic effects [15]. Growth stimulatory properties of mEGF are mediated by cAMP-independent protein kinases [16].

During plateau phase, DNA content of cell tends to increase. The possibility of a G_2 block preceding the onset of differentiation is under study by the flow cytofluorometric technique.

The flow cytofluorometric measurements were carried out by Dr. James C. Bartholomew, Laboratory of Chemical Biodynamics, University of California, Berkeley, CA. The authors acknowledge the advice of Dr. Alvin J. Cox of this Department in histological aspects of this work, and the technical help of Taiss Tomashevsky and Barbara Gray.

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